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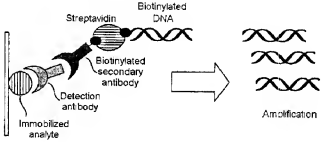
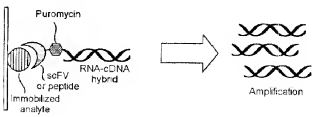
(54) Titre : SYSTEME D'ESSAI PERMETTANT DE DETECTER DES ANALYTES, UN PROCEDE DE REALISATION, ET SON UTILISATION

(54) Title: ASSAY SYSTEM FOR DETECTING ANALYTES AND A METHOD FOR THE PREPARATION THEREOF AND USE THEREOF

A: Immuno-PCR



B: RNA-protein fusions as ultrasensitive diagnostic system



(57) Abrégé/Abstract:
The invention relates to an in vitro assay for carrying out a sensitive and specific direct detection of analytes. According to the invention, polypeptide nucleic acid conjugates are advantageously used as a detector-amplifier system and the signals of these conjugates are amplified by means of PCR.

(57) **Abstract:** The invention relates to an *in vitro* assay for carrying out a sensitive and specific direct detection of analytes. According to the invention, polypeptide nucleic acid conjugates are advantageously used as a detector-amplifier system and the signals of these conjugates are amplified by means of PCR.

Assay system for detecting analytes and a method for the preparation thereof and use thereof

Description

The present invention relates to an assay system and to a method for simultaneous parallel detection of various analytes.

In the field of biological and biomedical diagnostics, proteins but also other substance classes such as biopolymers, organic compounds, tumor cells, viruses, bacteria or substances relating to the environment are identified and quantified by using mainly and preferably the principle of antigen-antibody reaction. The immune reaction which takes place on an insoluble support is made to appear by using systems in which the signal may be amplified, for example, by fluorescence, luminescence, radioactivity (RIA), enzymatic color reactions (ELISA) or electron-dense particles (e.g. colloidal gold). The antibody is coupled to the amplifier either directly or indirectly via a second antibody which is directed against the primary antibody and is labeled with the amplifier. Such immunological detection methods, "immunoassays", can make quantitative statements, as long as one of the reaction partners is coupled to a readily detectable labeling substance such that the immunological properties of the components are retained. The highly sensitive avidin/biotin system has proved a particularly advantageous coupling system (cf. e.g. Wilchek M., Bayer E.A. Avidin-Biotin Technology. Meth. Enzymol. V. 184, Academic Press, 1990).

The detection limit of such immunological methods is approximately 10^{-18} mol. Sano *et al.* succeeded in greatly increasing the sensitivity of immunological methods by combination with the extremely sensitive polymerase chain reaction (PCR for short) which allows detection of down to 10^{-22} mol (5×10^2 molecules) (U.S. 5,665,539; Sano, T., Smith, C.L., Cantor, C.R., *Science* 1992, *258*, 120-122). The "immuno-PCR" uses antibodies linked to a DNA marker to visualize the immune reaction. PCR amplification of said DNA makes it possible to detect even amounts of approx. 500 molecules, corresponding to a more than 1000-fold increase in the detection limit of standard protein analysis (cf. diagram in Figure 1A).

The capability of immuno-PCR with respect to analytical and biomedical/diagnostic questions was demonstrated in a number of printed publications (Maia, M., Takahashi, H., Adler, K., Garlick, R.K., Wands, J.R., *J. Virol. Methods* 1996, *62*, 273-286; Sann, P.P., Weiss, F., Samson, M.E., Bloom, F.E., Pich, E.M., *Proc. Natl. Acad. Sci. USA* 1995, *92*, 272-275; Suzuki, A., Itoh, F., Hinoda, Y., Imai, K., *J. Cancer Res.* 1995, *86*, 885-889; Sperl, J., Paliwal, V., Ramabhadran, R., Nowak, B., Askenase, P.W., *J. Immunol. Method.* 1995, *186*, 181-194; Niemeyer, C.M., Adler, M., Blohm, D., *Anal. Biochem.* 1997, *246*, 140-145). In said examples the antibodies were labeled with DNA indirectly via molecules which bifunctionally bind both antibodies and DNA. Sano *et al.* use a recombinant fusion protein comprising a protein A moiety and a streptavidin moiety, which is able to bind simultaneously the Fc part of the antibody and biotinylated DNA. However, the use of such a fusion protein is disadvantageous if the serum or tissue material to be analyzed contains IgG-containing components, since it binds unspecifically to said components. It is possible to use as an alternative the commercially available proteins streptavidin and avidin (Ruszicka, V., März, W., Russ, A., Gross, W., *Science* 1993, *260*, 698; Zhou, H., Fischer R.J., Papas, T.S., *Nucleic Acids Res.* 1993, 21, 6038-6039). They have in each case four binding sites for biotin, whereby a biotinylated antibody is linked to biotinylated DNA. However, the immobilized antigen has to be incubated successively with a biotinylated primary antibody, streptavidin and biotinylated DNA marker, since simple mixing of the components results in a complex mixture of all kinds of possible combinations. In addition, numerous washing steps are necessary in order to remove excess components and to prevent unspecific binding. If no biotinylated primary antibody is available, it is also possible to use a biotinylated secondary antibody but this further increases the complexity of the assay.

Surprisingly, it has been found now that polypeptide-nucleic acid conjugates are particularly suitable for detecting analytes. Here the amplifier (= nucleic acid) is coupled directly via a linker to the detector (= a protein or a peptide specifically binding to the analyte), thereby advantageously and substantially reducing the number of incubation steps required and thus also the complexity of the assay. In addition, parallel detection of a plurality of analytes at the same time is made possible in this way (multiplexing).

The invention therefore relates to an assay system comprising

(a) at least one immobilized analyte on an insoluble support, and

(b) a polypeptide detector adapted to said analyte,

(c) said polypeptide detector being conjugated to an amplifier via a linker.

It is therefore the object of the invention to provide such an assay system for direct detection of analytes together with a method and the use thereof.

In accordance with the invention, assay system means an in vitro assay with high-throughput quality, since a very high sample throughput is attained. A high sensitivity and sensitivity and also specificity are attained.

Proteins or peptides which bind and therefore detect the substance to be detected (analyte) with sufficient affinity are, for example, single-chain antibodies (scFv), natural binding partners or binders generated by combinatorial selection methods, which are preferably prepared by means of *in vitro* translation with provision of polypeptide-nucleic acid conjugates. In this context, reference is explicitly made to the disclosure in WO 98/31700. Preferably in WO 98/31700, proteins or peptides are covalently linked, during translation, to the RNA encoding them and thus inherently carry the genetic information for their synthesis (coupling of phenotype and genotype). In order to generate said RNA-protein conjugates, preferably puromycin, a puromycin derivative or another molecule which mimics the structure of loaded tRNAs is bound to the RNA via a linker. As soon as the translation reaches the end of the coding sequence, puromycin occupies the A site and is linked to the newly synthesized protein via an amide bond. Comparable techniques which can be used for the present invention are described for the skilled worker, for example, in DE 19646372C1, WO 98/16636, WO 91/05058, U.S. 5,843,701, WO 93/03172 or WO 94/13623. The thus prepared protein or peptide (= detector) in the polypeptide-nucleic acid conjugate enables via specific binding detection of the analyte, the genetic information of the detector being present in the conjugate in the form of RNA, which information is amplified according to the invention preferably by means of reverse transcriptase PCR (RT PCR for short), with conversion into a signal. Therefore, the RNA of the conjugate serves as an amplifier (signal amplification). Alternatively to this, it is possible to reverse transcribe the

RNA portion of the conjugate already prior to use in the immuno PCR, so that amplification of the nucleic acid requires only one PCR.

The method of the invention is carried out by first immobilizing at least one analyte to an insoluble support (e.g. plastic, ceramic, metal, glasses, crystalline materials or (bio)molecular filaments such as cellulose, structural proteins). If the analyte is a protein, said immobilization is preferably carried out directly in microtiter plates made of polystyrene, a material which has a high reproducible protein adsorption capacity, or, for example, on materials whose surfaces have been modified for covalent immobilization of proteins. In another embodiment, the analyte can be immobilized by specifically binding capture antibodies (sandwich immuno PCR). After incubating the analyte with the nucleic acid-protein conjugate and removing unspecifically bound material, the nucleic acid is amplified by means of RT PCR or PCR or via other suitable methods, for example primer extension (cf. Ruano, G., Lewis, M.E., Kouri, R.E., Anal. Biochem., 1993, 212, 1-6) or SDA (strand displacement amplification) (cf. Walker, G.T., Little, M.C., Nadeau, J.G. and Shank, D.D., Proc. Natl. Acad. 1992, Sci 89, 392-396 and Walker, G.T., Fraiser, M.S., Schram, J.L., Little, M.C., Nadeau, J.G. and Malinowski, D.P., Nucleic Acids Res. 1992, 20, 1691-1696). The amplified products are detected either directly in the reaction vessel or after fractionation, for example by means of gel electrophoresis. For detection, either marker substances are incorporated into the nucleic acid during amplification or an indirect labeling is carried out following amplification, for example by hybridizing labeled nucleic acid probes. Examples of labeling methods are chemical, enzyme, protein, hapten, radioisotopic, non-radioisotopic, chemiluminescent and fluorescent labeling.

In conventional immuno PCR, the detector antibodies are labeled indirectly with the biotinylated DNA via biotin-binding molecules such as streptavidin or protein A/streptavidin fusions. These methods require numerous incubation steps for the addition of up to three reporter reagents and, in addition, a large number of washing steps in order to remove excess and unspecifically bound reagents. The covalent linkage of the nucleic acid (= amplifier) to the detector greatly reduces the required number of incubation and washing steps and thus also the time and complexity of the assay.

In addition, it is possible, in contrast to conventional immuno PCR, to assay a plurality of analytes simultaneously. The development of methods for parallel detection of a plurality of analytes in a sample is regarded as an important aim in biomedical diagnostics, owing to the reduction in costs and time. Moreover, the need for detecting whole groups of analytes steadily increases, not only in the clinical routine but also, for example, in environmental diagnostics. Up until now, multianalyte assays have been carried out by means of combinations of radioisotopes (Morgan, C.R., *Proc. Soc. Exp. Biol. Med.* 1966, *123*, 230-233), fluorescent markers (Kakabakos, S.E., Christopoulos, T.K., Diamandis, E.P., *Clinical Chem.* 1992, *38*, 338-342), chemiluminescent markers (Yamamoto, K., Higashimoto, K., Minagawa, H., Okada, M., Kasahara, Y., *Clinical Chem.* 1991, *37*, 1031) or enzyme-labeled antibodies (Kricka, L.J., *Clinical Chem.* 1992, *38*, 327), but overlapping of the signals of various markers and differences in the signal intensity at various analyte concentrations greatly impair quantification and sensitivity. In contrast, nucleic acids are ideally suited as markers for multianalyte assays, since various DNA molecules can be distinguished from one another accurately and quantitatively both on the basis of their size and on the basis of their sequence so that the use of nucleic acids makes it possible to produce an almost unlimited number of labels.

All activated substances are suitable for the assay system and method of the invention, in particular substances such as diagnostics, biopolymers, biological domains such as antigens and haptens, hormones, cytokines, pheromones, secondary metabolites, pharmaceuticals, opiates, nucleic acids and also low molecular weight to macromolecular organic compounds (e.g. herbicides or pesticides).

Examples:

The following example describes detection of an immobilized antibody against c-myc with the aid of a fusagene and subsequent PCR amplification.

A 114-mer DNA template (5'-ATGGTGAGCAAGGGCGAGGAGC AAAAGCTTATTTCTGAAGAGGACTTGCTTAAGGGAAACTCACAGGAAG CTGTGTTAAAGTTGCAAGACTGGGATGCACAAGCACCAAAAGCT-3') coding for the amino acid sequence of the c-myc epitope is prepared by means of solid phase synthesis in an oligonucleotide synthesizer and then

amplified by PCR using 1 µM primers 5'-TAATACGACTCACTATAGGGACAATTACTATTTACATTACAATGGTGAG CAAGGGCGAGGAG-3' and 5'-AGCTTTTGGTGCTTGTGCATC-3' and also 0.02 U/µl Taq polymerase (Promega) in 10 mM Tris-HCl pH 9.0, 50 mM KCl 0.1% Triton X-100, 2.5 mM $MgCl_2$ and 0.25 mM dNTPs. The 5' primer introduces a T7 promoter region and a region from the 5' untranslated sequence of the tobacco mosaic virus genome as translation initiation site. The double-stranded PCR product is transcribed into RNA by using the Megashortscript transcription kit (Ambion) according to the manufacturer's instructions and said RNA is then purified via Microspin Sephadex G25 columns (Pharmacia). The puromycin linker is ligated by adding 1 nmol of linker (5'-A_{27}CC-puromycin-3') and 1 nmol of splint (5'-TTTTTTTTTTNAGCTTTTGGTGCTTG-3') to 1 nmol of RNA and denaturing the mixture at 95°C for 3 min. Addition of 10 × ligation buffer (300 mM Tris-HCl pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP) and annealing at room temperature for 15 minutes are followed by ligation at room temperature for 4 h. The ligated RNA is separated from unligated RNA via a denaturing polyacrylamide gel and eluted from the gel in 0.3 M NaOAc pH 5.2 overnight. 50 pmol RNA are used for generating the peptide-RNA conjugate by using the Retic Lysate in vitro translation kit (Ambion). This is followed by purification via oligo-dT cellulose. For this purpose, the translation mixture is incubated with 100 µl of oligo-dT cellulose (Pharmacia) in 100 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 1 M NaCl and 0.25% Triton X-100, at 4°C for 1 h and bound fusion product is subsequently eluted with ddH_2O. cDNA is synthesized by adding a 5-fold excess of splint to the RNA-peptide conjugates. Reverse transcription is carried out using 0.007 U/µl Superscript II reverse transcriptase (Gibco BRL) in 25 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mm DTT and 0.5 mM dNTPs.

Decreasing amounts of the monoclonal antibody 9E10 against c-myc (Chemicon) are incubated for immobilization in 50 µl PBS (137 mM NaCl, 2.7 mM KCl, 8 mM NaH_2PO_4, 2 mM Na_2HPO_4) in Top Yield□ reaction vessels (Nunc) at room temperature overnight. After washing three times with 200 µl each of PBS, excess binding sites are blocked with in each case 200 µl of 4.5% skimmed milk powder, 0.1 mM EDTA pH 8.0, 1 mg/ml salmon sperm DNA and 0.2% sodium azide at room temperature for 1 h. After washing three times with in each case 200 µl of TEPBS (0.05% (v/v) Tween-20, 100 mM EDTA, 137 mM NaCl, 2.7 mM KCl, 8 mM NaH_2PO_4,

2 mM Na_2HPO_4), the samples are incubated with in each case 1×10^{-15} mol myc fusagene in 50 µl of TEPBS at room temperature for 1 h. After washing again three times with in each case 200 µl of TEPBS, the PCR amplification is carried out in 50 µl reaction volumes using 1 µM primers 5'-TAATACGACTCACTATAGGGACAATTACTATTTACATTAC AATGGTGAGCAAGGGCGAGGAG-3' and 5'-AGCTTTTGGTGCTTGTGC ATC-3' and 0.02 U/µl Taq polymerase (Promega) in 10 mM Tris-HCl pH 9.0, 50 mM KCl 0.1% Triton X-100, 2.5 mM $MgCl_2$ and 0.25 mM dNTPs for 30 cycles (1 min 95°C, 1 min 55°C, 1 min 72°C). The samples are analyzed on a 2% strength agarose gel.

Description of the figures:

Figure 1A depicts the principle of immuno PCR

Figure 1B depicts the principle of the invention.

Patent claims

1. An assay system comprising
   (a) at least one immobilized analyte on an insoluble support, and
   (b) a polypeptide detector adapted to said analyte,
   (c) said polypeptide detector being conjugated to an amplifier via a linker.

2. The assay system as claimed in claim 1, wherein (c) is a polypeptide-nucleic acid conjugate.

3. The assay system as claimed in claim 1 or 2, wherein the linker is puromycin, a puromycin derivative or a binding modified t-RNA.

4. The assay system as claimed in any of claims 1-3, characterised in that the amplifier is an RNA, and the signal is amplified by means of PCR.

5. The PCR as claimed in the preceding claim, as RT PCR method or primer extension method or a strand displacement amplification.

6. The assay system as claimed in claim 1, characterised in that the analyte is selected from the group consisting of diagnostics, biopolymers, biological domains such as antigens and haptens, hormones, cytokines, pheromones, secondary metabolites, pharmaceuticals, opiates, nucleic acids and also low molecular weight to macromolecular organic compounds (e.g. herbicides or pesticides).

7. The assay system as claimed in claim 1, characterised in that the analyte is an antigen and the polypeptide detector is an antibody, preferably a single-chain antibody.

8. A method for detecting analytes, characterised in that
   (a) at least one analyte is immobilized on an insoluble support and
   (b) binds to an adapted polypeptide detector which is conjugated to an amplifier via a linker and,

(c) after washing,

(d) the signal is amplified by means of PCR.

9. The use of polypeptide-nucleic acid conjugates for parallel detection of various analytes.

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Fig. 1A: Immuno-PCR

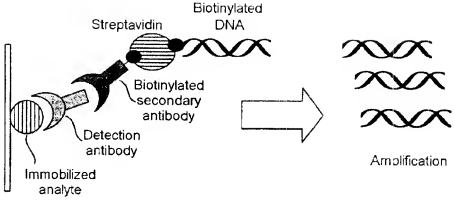
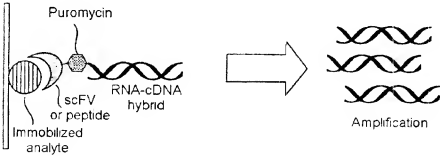
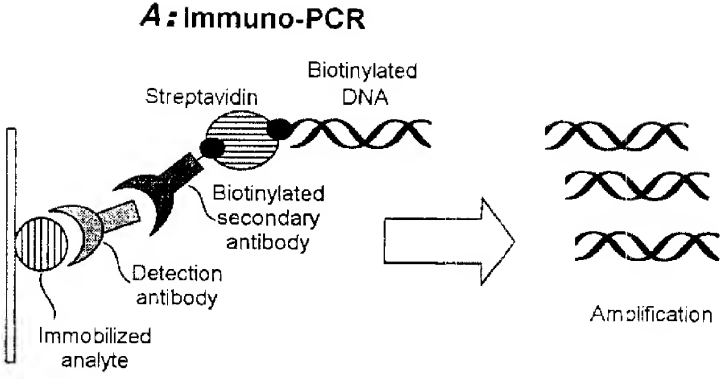
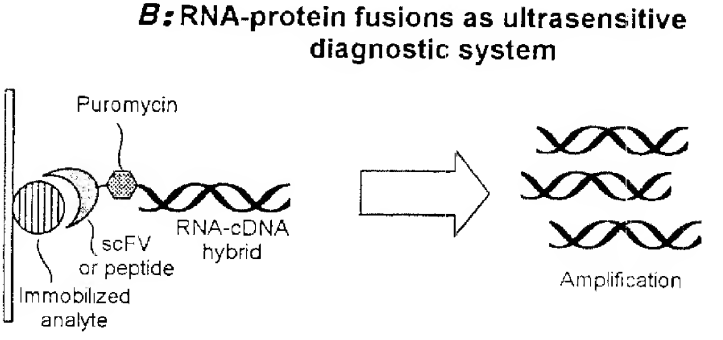


Fig. 1B: RNA-protein fusions as ultrasensitive diagnostic system




A: Immuno-PCR
Biotinylated DNA
Streptavidin
Biotinylated secondary antibody
Detection antibody
Immobilized analyte
Amplification


B: RNA-protein fusions as ultrasensitive diagnostic system
Puromycin
RNA-cDNA hybrid
scFV or peptide
Immobilized analyte
Amplification